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The impact of the polymorphism of *PACRG* and *CD80* genes on the development of different stages of tuberculosis infection

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Running Title: The polymorphism of *PACRG* and *CD80* genes and tuberculosis

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What's Known (word count 50 words up to two bullets)
• After infection by *Mycobacterium tuberculosis,* within 2 years 5% of individuals develop clinical manifestations of the primary tuberculosis. 5-10% of those infected during the course of their life develops secondary tuberculosis. • There is an assumption that different genetic factors affect the risk of primary tuberculosis and secondary tuberculosis but genetic studies considering these stages of the disease separately are limited.
What's New (word count 50 words up to two bullets)
• In our study, for the first time, we identified an association between polymorphisms of *PACRG* (rs10945890) and *CD80* (rs1880661) genes and the development of different stages of tuberculosis infection. These genes have never been studies before regarding TB. • We found a novel proof that predisposition to different forms of TB infection are under control of different genetic factors of the host.

Abstract

Background: Tuberculosis (TB) is one of the most significant health care problems worldwide. Host genetics play an important role in the development of TB in humans. The disease progresses through several stages, each can be under the control of different genes. The precise genes influencing different stages of the disease are not yet identified. The aim of the current study was to analyse associations between primary and secondary TB and polymorphisms of novel TB susceptibility candidate genes – *CD79A, HCST, CXCR4, CD4, CD80, CP, PACRG,* and *CD69.*

Materials and methods: A total of 362 TB patients from Siberian region of Russia were studied, including 130 cases with primary TB and 232 cases with secondary TB, as well as 445 healthy controls. The study was performed at the Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, Russia, between July 2015 and November 2016. Genotyping was carried out using MALDI-TOF mass-spectrometry and PCR-RFLP. Association between the SNPs and TB was assessed using logistic regression adjusting for covariates (age and gender). Multiple testing issue was addressed via experiment-wise permutation approach. Statistical significance threshold was $p<0.05$ for permutation p-values. Analysis was done in R 3.2 statistical software.

Results: An association was established between the rs1880661 variant of *CD80* gene and secondary TB and rs10945890 variant of *PACRG* gene and both primary and secondary TB. However, the same allele of *PACRG* appeared to be both a risk factor for reactivation (secondary TB), while being protective against primary infection.

Conclusion: The results suggest that *CD80* and *PACRG* genes are associated with susceptibility to different forms of TB infection in the Russian population.

Keywords: Tuberculosis, Polymorphism, Single Nucleotide Polymorphism, *CD80, PACRG*

Introduction

Tuberculosis (TB) remains one of the most dangerous infectious human diseases. In 2015, 10.4 million new TB cases we estimated and 1.8 million people (including 0.4 million people with HIV) died as a result of the disease.[1] Importantly, infection of a human with *M. tuberculosis* is not enough for TB to progress into a clinical disease. Only about 10% of infected cases develop active disease, while the rest remain latently infected or completely get rid of bacteria. The outcome of the infection is dictated by such factors as the environment, virulence of bacterial strain, infection load, as well as individual immune system features of the host which are strongly genetically determined.

The involvement of certain regions of the human genome in the susceptibility to TB has been the target of active research over the past decades. As the result of candidate genes studies, many genes encoding enzymes with immune functions have been identified, some of them have been shown to have a "major gene" effect on TB susceptibility.[2] Thus far, 10 genome-wide association studies (GWAS) for TB have been published that identified more than 20 genes associated with the disease, including *ASAP1, AGMO, FOXP1* involved in the functioning of macrophages and dendritic cells.[3]

Also, as the result of the studies of atypical familial mycobacteriosis, rare mutations in *IL12B/IFNG* genes responsible for anti-infectious immunity were revealed. The mutations cause the development of severe, and usually lethal disease in response to non-pathogenic or mildly pathogenic bacteria including *M. bovis*, *M. avium*, and *S. enterica*. These studies played an important role in the identification of genes involved in immune response against mycobacteria.

Despite the remarkable achievements in understanding the pathogenesis of TB, there are substantial unresolved issues in diagnostics, prophylaxis and treatment of the disease. Lack of understanding of the mechanisms of reactivation of latent infection - which creates a huge reservoir of the dangerous disease distribution for many years, is especially worrying.[4] The

existent diagnostics tools are based on the analysis of *M. tuberculosis* patients' sputum and X-ray examination; however, their disadvantages include a delay in the finding of bacteria in sputum, thus delaying the treatment.[5] Also, there is a lack of information about molecular genetic mechanisms by which susceptibility to TB converts to the disease, in turn, resulting in an slow progress in the development of effective treatment and prevention strategies.

More recently, systems biology and bioinformatics approaches are being used for the discovery of novel anti-tuberculosis drug targets. In particular, bioinformatics strategies are directed towards the studies of "host-pathogen" interactions[6-7], because it is known that the success of *M. tuberculosis* is driven by its capability to modify human immune response[8], with different strains of bacteria being able to induce various patterns of the host immune response.[9]

Likely, an approach based on the revelation of functional interactions between genes/proteins involved in gene networks would improve our understanding of the nature of the dynamic reaction to infection and would help establish most important molecular participants in the disease development. Thanks to the network approach describing protein-protein interactions for the genes differentially expressed in TB patients, a pattern of genes called "common core" for the disease was discovered including genes important in immune response, such as *STAT1*, *PLSCR1*, *C1QB*, *OAS1*, *GBP2* and *PSMB9*.[10]

In our previous study, we carried out a reconstruction of associative network for TB and revealed novel candidate genes including *CD4*, *CD69*, *CD79*, *CD80*, *MUC16*, *HCST*, *ADA*, *CP*, *SPP1*, *CXCR4*, *AGER*, and *PACRG*.[11] As a follow up, in the current study we analysed regulatory polymorphisms for the genes from the associative network to assess their pathogenetic significance for different stages of TB infection.

Material and methods

The study was approved by the Ethics Committee of the Research Institute of Medical Genetics of Tomsk NRMC and signed informed consent was obtained from all participants. The study was performed at the Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, Russia, between July 2015 and November 2016.

The diagnosis of TB was established on the basis of sputum microscopy data with mandatory X-ray examination of the lungs to determine the form of the disease and the prevalence of a specific process. HIV-positive patients were excluded. Control group included healthy individuals without history of TB. The participants were predominantly Russians settled in the city of Tomsk or Tomsk Region, West Siberia, Russia. The demographic and clinical data for each patient were collected.

DNA samples were retrieved for 362 TB patients and 445 healthy controls from the DNA Bank of the Research Institute of Medical Genetics of Tomsk NRMC (Table 1). The sample of TB patients included 130 cases with primary TB and 232 with reactivation. The control group for primary TB was deliberately older as compared to the cases (16.6±15.0 vs 39.5±17.0) to ensure that the control individuals were not affected by TB up until adulthood.

For the genotyping, we chose 14 single nucleotide polymorphisms (SNPs) in 8 genes in which regulatory capacity was established using data from RegulomeDB database (Table 2). The database classifies SNPs into classes according to the combined status of overlap with functional categories such as transcription factor binding sites, DNase I hypersensitivity and promoters, and assigns respective score from 1 to 6 with the smaller score meaning higher functional impact of a SNP (http://regulomedb.org/).

Genotyping was carried out using MALDI-TOF mass-spectrometry and PCR-RFLP. For MALDI-TOF mass-spectrometry we used iPLEX GOLD kits (Agena Bioscience) and MassARRAY Analyzer 4 (Seqeunom). Genotype calls were done automatically by MassARRAY Typer 4 software. PCR-RFLP was carried out using custom primers and specific

restriction endonucleases (Fermentas and Sibenzyme) (Table 3). All analyses were carried out in the "Medical Genomics" Core Facilities of the Research Institute of Medical Genetics.

Association between the SNPs and TB was assessed using logistic regression adjusting for covariates (age and gender). Additive, dominant and recessive genetic models were tested. In dominant model, rare allele homo- and heterozygotes are tested against common allele homozygotes. In the recessive model, common allele homo- and heterozygotes were tested against rare allele homozygotes. The additive model corresponds to a trend test for the genotypes with the genotypes coded as 0, 1, or 2 to reflect the minor allele counts. The best model was chosen using Akaike Information Criterion. The SNP effects were quantified with the odds ratio (OR) and 95% confidence intervals. Multiple testing was addressed using experiment-wise permutations. Models with permutation $p<0.05$ were considered statistically significant. Statistical analysis was carried out in R 3.2 statistical software.

Results

We chose new candidate genes of special interest based on the results of our previous study: *CD4, CD69, CD79, CD80, MUC16, HCST, ADA, CP, SPP1, CXCR4, AGER,* and *PACRG*.[11] To the best of our knowledge, these genes have never been studied with respect to TB. To select SNPs in these genes, we took into account such parameters as 1) localization in the 5 'region of the gene; 2) the global frequency of the minor allele ≥5%; 3) SNPs with high confidence of functional consequence in genes region using RegulomeDB. Accordingly, we selected for the genotyping 14 SNPs in 8 genes (*CD4, CD69, CD79, CD80, HCST, CP, CXCR4,* and *PACRG*; Table 2).

In the control group as well as in patients group, all SNPs met Hardy-Weinberg equilibrium expectation. The rs75343219 SNP in *CD69* was monomorphic in Russians and excluded from subsequent analysis.

An association was established between TB and the rs1880661 polymorphism in *CD80* gene (Table 4). When primary and secondary TB were considered separately, an association was found between this polymorphism and secondary TB only. The prevalence of the rs1880661*C allele of *CD80* gene in patient with secondary TB was 40.9%, in patient with primary TB it was 44.7% and in the control group it was 46,8%.

Also, the rs10945890 variant in *PACRG* was associated both with primary and secondary TB; however, there was a recessive effect of the polymorphism for primary TB and dominant effect for secondary TB. The same allele rs10945890*C of the gene *PACRG* was associated with the decreased risk of primary TB (OR=0.26 [0.04;0.89]; p=0.03), but with the increased risk of reactivation (OR=1.47 [1.02;2.13]; p=0.04). In patients with primary TB, the frequency of the rs10945890*C allele of *PACRG* gene was 20.5%, in patient with secondary TB it was 28.6% and in the control group it was 25.6%.

Discussion

We carried out an analysis of association between different stages of TB infection and potential regulatory SNPs in *CD4*, *CD69*, *CD79*, *CD80*, *HCST*, *CP*, *CXCR4*, and *PACRG* genes. Most of the proteins encoded for by these genes are involved in immune signaling and are responsible for the effectiveness of immune reactions to the invasion of the pathogen. Polymorphisms in *CD80* and *PACRG* genes were found to be associated with different stages of TB in Russians.

CD80 gene encodes a transmembrane receptor, a co-stimulator for antigen presentation by macrophages and dendritic cells. Its expression is reduced in mycobacterial infection, the mechanism by which mycobacteria supresses adaptive immune response.[12] According to our previous data, *CD80* is the most promising candidate gene out of the lot from TB associative network.[11] We studied three SNPs in this gene (rs59569688, rs3915165, rs1880661) and found that rs1880661 is associated with secondary TB. This polymorphism is an expression

quantitative trait locus (eQTL) and influences the expression of *CD80* and *ADPRH* in a tissue-specific manner (Figure. 1). The SNP is associated with differential expression of *CD80* in dendritic cells before and after mycobacterial infection.[13] The association between TB and this SNP was established for the first time in the current study; however, there are other polymorphisms in *CD80* associated with immune-mediated diseases, such as celiac disease (rs11712165)[14] and primary biliary cirrhosis (rs2293370).[15]

The *PACRG* gene encodes parkin co-regulated protein and located on 6q26 the cluster with related gene *PARK2*. These genes have a common regulatory region and are involved in ubiquitin-mediated protein degradation. They were found to be important for susceptibility to diseases caused by *M. ulcerans* and *M. leprae*.[16] The variant of *PACRG* associated with TB in the current study (rs10945890) has never been studied for association with TB or other diseases. It is also challenging to explain why the same allele of the gene is associated with the decreased risk of primary TB (OR=0.26 [0.04;0.89]; p=0.03), but increases the risk of reactivation (OR=1.47 [1.02;2.13]; p=0.04). Functional studies as well as replication in other populations will be required to delineate this.

No other studied genes were found to be associated with TB in the current study; however, their analysis in other populations may still be fruitful given their functional importance in TB pathogenesis.

The *CD69* gene located on 12p13.31 and encodes type II transmembrane glycoprotein. Increased expression of the *CD69* gene is observed in TB patients.[17] Polymorphism rs4763879 in this gene was found to be associated with type I diabetes.[18]

The *CD4* gene (12p13.31) encodes the membrane glycoprotein of T-lymphocytes which plays an important role in T-helper cell activation. The deficit of CD4+ T cells promotes susceptibility to *M. tuberculosis* infection.[19] Except for the current study, polymorphisms of *CD4* gene have never been studied in TB susceptibility.

The *CD79A* gene encodes Ig-α protein expressed in B-lymphocytes. The protein is essential for immune pathogenesis of TB.[20] The gene located on 19q13.2 and is associated with cancer[21] however there are not studies of the polymorphisms of *CD69* gene and susceptibility to TB.

The *HCST* (*DAP10*) gene located on 19q13.12 encodes a transmembrane signaling adaptor containing the YxxM motif in its cytoplasmic domain. The expression of *HCST* gene was found repressed during late stages of infection in nonhuman primates infected by *M. tuberculosis*.[22] This gene is of interest for the study of different stages of TB infection but the polymorphisms of this gene have never been studied in this respect.

The *CP* gene encodes ceruloplasmin, a metalloprotein which binds up to 95% of blood cuprum. Copper along with other microelements are important in protecting against pathogenic microorganisms[23], which underlies the antibacterial function of ceruloplasmin. Ceruloplasmin is an acute phase protein; its concentration, along with cuprum ions levels are elevated in lung TB patients.[24] Defects in the *CP* can lead to a disruption of the binding and transport function of ceruloplasmin and, as a result, an increase in sensitivity to intracellular pathogens, such as mycobacteria. No studies of association between this gene variants and TB are available.

The gene *CXCR4* (2q22.1) encodes chemokine (CXC motif) receptor 4, CXCR4 is involved in angiogenesis induced by granuloma.[25] The polymorphisms of gene *CXCR4* rs2680880 associated with overall survival from colorectal brain metastases diagnosis[26], another SNP of this gene rs953387 links with juvenile idiopathic arthritis, an autoimmune disease.[27]

Thus, the above genes are important for an effective immune response to the invasion of the pathogen, but they have not been extensively studied in TB. Thus, our study provides the first glance at these genes with regards to TB.

The majority of genetic studies of TB are focused on establishing associations between the disease per se and genetic variants. However, studies considering clinical forms or stages of

the disease are limited. The current study was carried out to reveal genetic factors associated with various stages of TB.

In contemporary studies of TB, the major question is why immunity is able to control the infection in primary contact, but cannot prevent reactivation. High resistance to primary TB actually predisposes to the development of secondary TB.[28] The majority of immunocompetent people would develop delayed hypersensitivity activating T-cells and Th1-immunity which effectively controls primary TB. However, this process has little effect on secondary TB, also neither immunization, nor natural infection result in immunity to secondary TB.[29] This means that mycobacteria employs an effective strategy to avoid host immune response or even benefit from it.[30]

Recently, it has been noted that the immune response characterised by elevated activity of CD4+ T-cells and increased levels of IFN-γ causes the development of secondary TB thus contradicting with a widely accepted view that impaired immunity leads to reactivation of latent infection.[31] Even though the risk of TB is increased when the immunity weakens, the disease aetiology in individuals with impaired immunity differs from the disease aetiology in immunocompetent people. The development of the disease in immunocompromised people is caused by an uncontrolled proliferation of the bacteria, while in individuals with healthy immune system, it is a damage of lung tissue that causes the development of active disease.[32] These different mechanisms may be the basis for clinical heterogeneity of TB, therefore different genes can be involved. Thus, primary and secondary TB can be controlled by different host genes[32-33], which is supported by the results of the current study.

The advantage of the current study is the focus on the analysis of novel candidate genes and stratified analysis according to the stages of TB infection. However, the study has a limitation – the control group was significantly older than the cases. However, this was done to

avoid possible risk of TB in subsequent life of young individuals, if taken as control. Another limitation is the lack of replication sample. Thus, our findings require independent validation.

Conclusion

In summary, our data suggest that the studied polymorphisms in *CD80* and *PACRG* genes affect susceptibility to different stages of TB infection (primary and secondary TB) in Russian patients. If replicated in independent samples, the mechanisms of the associations are to be disclosed in experimental studies. However, given that we analyzed SNPs from regulatory regions of the genes, the mechanisms are likely related to modulation of the gene expression.

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Table 1 – Demographics of the studied individuals

Group	Number	Mean age±SD, years	p
Tuberculosis*	362	29.0±17.4	4 x 10-17
Females	125	23.0±16.3	
Males	232	32.3±17.1	
Primary tuberculosis	130	16.6±15.0	1.4 x 10-35
Females	63	14.3±13.0	
Males	67	18.8±16.3	
Secondary tuberculosis	232	36.1±14.5	0.007
Females	63	31.7±14.6	
Males	165	37.8±14.1	
Healthy control	445	39.5±17.0	
Females	273	38.3±17.2	
Males	172	41.5±16.7	

* Primary and secondary TB together; p value for Student's t-test for comparison between groups of patients and healthy individuals

Table 2 – List of studied SNPs with localization and Regulome DB scores.

Gene	Location	SNP	MAF	Marker position	Score in Regulome DB
CXCR4	2q22.1	rs12691874	0.31 (A)	2:136122904	2a
CD80	3q13.33	rs59569688	0.18 (T)	3:119559065	2b
CD80	3q13.33	rs3915165	0.23 (T)	3:119560504	2b
CD80	3q13.33	rs1880661	0.34 (G)	3:119560001	2b
CP	3q23-q25	rs7623663	0.16 (T)	3:149224171	2b
PACRG	6q26	rs12211969	0.13 (G)	6:163312136	2b
PACRG	6q26	rs58627325	0.14 (A)	6:163309605	2a
PACRG	6q26	rs6455894	0.20 (A)	6:163311988	2a
PACRG	6q26	rs10945890	0.30 (C)	6:163308974	2b
CD69	12p13.31	rs75343219	0.076 (G)	12:9761162	2b
CD4	12p13.31	rs2855534	0.47 (G)	12:6789355	2b
CD4	12p13.31	rs7296859	0.25 (C)	12:6784998	1f
CD79A	19q13.2	rs10417985	0.40 (T)	19:41873065	2b
HCST	19q13.12	rs11878547	0.09 (C)	19:35902284	2b

Table 3 - Sequences of primers and methods of genotyping.

ID SNP	Primer Forward 5'	Primer Reverse 5'	Primer Extention	Methods, restriction endonuclease and fragments
rs10417985	ACGTTGGATGACTTGCCAGATATCCCACAG	ACGTTGGATGTCTTTTCTGAGGCACAGAGC	gggtGAGTGGCTAGGTCCAGG	MALDI-TOF
rs11878547	ACGTTGGATGTCTTCTCAGCGTTTCATGCC	ACGTTGGATGGTAGGGCCAAGAAAATTTGC	ctgtCCAAGAAAATTTGCTGATTAAATG	MALDI-TOF
rs12691874	ACGTTGGATGGGTGACCTCAGACAGCTATA	ACGTTGGATGAAACTTGACAGTCCACAGGG	caggCCACAGGGCTCTAGG	MALDI-TOF
rs2855534	ACGTTGGATGTGCCATCTTTTTCTTGCCGC	ACGTTGGATGGAAAATGCCAAAGTCAAGGG	gggtCTTAACAGTGGCAGTGACA	MALDI-TOF
rs59569688	ACGTTGGATGAAAGAGACTTATTCACCAG	ACGTTGGATGAGACTGTGGTGAGCTATGGT	aAGAATTTGTTTTTCTTAAGATAGAAT	MALDI-TOF
rs7296859	ACGTTGGATGTTGACTTCCAGGCCACAGAC	ACGTTGGATGTTTGCAGATTCCAGACCCGA	ccttaCACAGACTCACAGAGCTG	MALDI-TOF
rs7623663	ACGTTGGATGTTGTGGTAGTTACTCTTCTC	ACGTTGGATGCCCCTCTCCCTCCTATTTAA	GCATGTGGCAGGAAGT	MALDI-TOF
rs12211969	ACGTTGGATGGGGTTTATGCAATGGGCTC	ACGTTGGATGTAGGCATGAAAGAGGTGGAC	TGCAATGGGCTCTGTCCT	MALDI-TOF
rs3915165	ACGTTGGATGGGGTTTATGCAATGGGCTC	ACGTTGGATGTAGGCATGAAAGAGGTGGAC	TGCAATGGGCTCTGTCCT	MALDI-TOF
rs58627325	ACGTTGGATGTTCAAGCTTCCGAAAGCAGG	ACGTTGGATGCTGAGTGAATCAGGAAATGG	aAGGAAATGGTTAAGAGGTGA	MALDI-TOF
rs6455894	ACGTTGGATGTGGTCTATTCAGCTCTTGAC	ACGTTGGATGGTCACTCATAAATGGTGCCT	GTGCCTTTTGTCGGCATAT	MALDI-TOF
rs75343219	ACGTTGGATGAGTGGGATTTTCCCAGACTC	ACGTTGGATGACTTAGATTATGCTGTCTCC	ggtaCTGCCTTAAATTTCTAGAAAAC	MALDI-TOF
rs1880661	AAGATGGTGGGATTCAGAGG	TGTTTCTGTGCTGGTCTCAA	-	***Bme18** TT 160 TC 160+122+38 CC 122+38I
rs10945890	CCAATCAGAAGAAAGCCAGC	TCTCGCTGAAGCAACACTGA	-	***HinfI** CC 219 CT 219+122+97 TT 122+97

*PCR-PLRF

Table 4 – Statistically significant models for association between tuberculosis and the polymorphisms of *PACRG* (rs10945890) and *CD80* (rs1880661) genes.

Sample	**SNP**	**Best model**				
		Risk allele	%	**Effect**	**OR [95% CI]**	**P-perm***
Tuberculosis	rs1880661	C	41.6	Dominant	0.68 [0.48;0.96]	0.03
Primary tuberculosis	rs10945890	C	20.5	Recessive	0.26 [0.04;0.89]	0.03
Secondary tuberculosis	rs10945890	C	28.6	Dominant	1.47 [1.02;2.13]	0.04
	rs1880661	C	40.9	Dominant	0.67 [0.46; 0.97]	0.04

* Experiment-wise permutation p-value

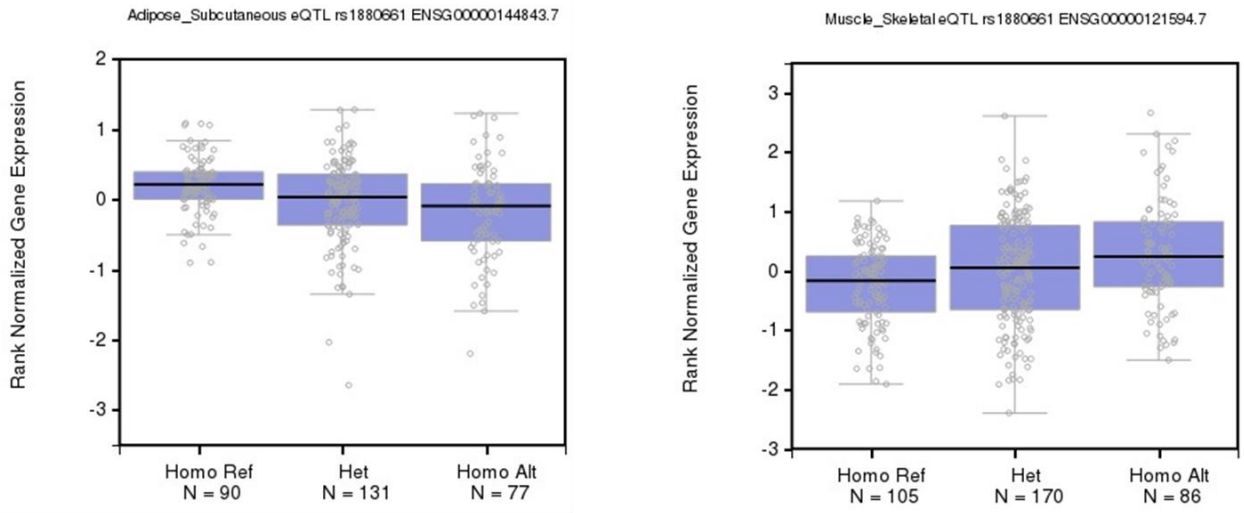

Figure 1 – GTEx box plot of *CD80* and *ADPRH* genes expression for the cis-eQTL rs1880661.

Box plots of gene expression profiles for *CD80* (ENSG00000121594.7) in muscle tissue and *ADPRH* (ENSG00000144843.7) in adipose tissue expression by genotype (data of the GTEx project portal (http://www.gtexportal.org/home/)). Reference allele (risk allele) = C.